

Changes in the Expression of p21, p27 and p53 Following Beta-1 Integrin Blocking in Murine Clara Cells Cultures

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INTRODUCTION

Integrins are the primary receptors used by cells to interact with extracellular matrix. Both ECM and integrins play an important part in the survival, proliferation and death of cells. When an injury occurs in lung epithelial cells signals through the integrins could significantly effect whether disease or repair occur.

Laminin attaches to a number of integrins including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_4$ [1-4]. Various β_1 and β_4 integrin complexes were shown to be involved in the activation of a number of cell cyclin inhibitors [5-7].

$\alpha_6\beta_4$ was shown to activate p53 function in carcinoma cells [8] and p53 was found to inhibit $\alpha_6\beta_4$ integrin survival signalling [9]. The β_4 integrin cytoplasmic domain was shown to be involved in the activation of p21 pathways of growth arrest and apoptosis [5]. T cell proliferation in the human thymus was found to be controlled via $\alpha_6\beta_4$ by laminin 5 Vivinus-Nebot *et al.* [4].

$\alpha_5\beta_1$, a fibronectin binding integrin, was found to negatively regulate cell growth in the absence of attachment to fibronectin [10]. $\alpha_5\beta_1$ was reported to be involved in the expression of tumour suppressor p16^{INK4a} upon loss of anchorage (anoikis) in a number of human cancer cells [11] and also supports survival of cells by up-regulating Bcl-2 expression [12]. Trypsin was also found to stimulate integrin $\alpha_5\beta_1$ dependent adhesion to fibronectin [13]. $\alpha_6\beta_1$ was shown to be an essential step accompanying the neoplastic transformation of hepatocytes [14].

MATERIALS AND METHODS

Clara cell isolation and culturing: Mice (C3H/He strain or p21 ko mice either male or female, between 4 to 8 weeks old) were sacrificed by lethal intraperitoneal injection of 0.5ml pentobarbitone (SagatalTM). p21 ko mice were kindly provided by Dr. Philip Leder, Harvard Medical School,

Boston. Clara cells were isolated and cultured as previously described [15].

Once isolated, cells were plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with appropriate laminin (50 μ g/mL) and incubated at 37°C, 5% CO₂/air. Cells were allowed to attach overnight after which the medium was replaced to remove dead and unattached cells. Medium was subsequently replaced every 2 days. Cells were usually fixed at days 1, 3 and 5 by methanol at -20°C.

Culture medium: Mouse bronchiolar cells were cultured in a 1:1 mixture of Hams F12 (Gibco) and M-199 medium (Gibco) supplemented with 2 mM L-glutamine, 10 μ g/mL insulin, 5 μ g/mL transferrin, 100 ng/mL hydrocortisone, 10 ng/mL EGF, 0.1 ng/mL retinyl acetate and Pen/Strep [16].

Integrin blocking experiments: Clara cells from both wt and p21 ko mice were cultured on Laminin at a concentration of 50 μ g/mL and incubated with either a β_1 blocking antibody (Hm β_1) or with a cytokeratin 8 antibody as a control. Both antibodies were used at a concentration of 10 μ g/mL. The β_1 integrin antibody was obtained as a gift from Prof. Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan. This antibody is now commercially available from PharMingen (Catalogue No. 553837). The Hm β_1 antibody has been shown previously to react with mouse β_1 and has an inhibitory effect on cell adhesion to ECM proteins this is a functional β_1 blocking antibody [17,18]. The cytokeratin 8 antibody (10526 ICN) does not have an inhibitory function.

Immunocyto/histo-chemistry: Slides were equilibrated in TBS for 5 min. Please note that the work slides refer to cytopins, tissue sections as well as cultured cells. The slides were blocked with an adequate serum in which the secondary antibody was raised. Primary antibody at a

Table 1: List of antibody used with details of concentration used, supplier and catalogue number

Antibody	Concentration	Supplier	Catalogue number
p21	1/10	DAKO	M7207
p53	1/100	Vector Lab	NCL-p53-CM5p
p27	1/200	SIGMA	P2092

concentration specified in the Table 1 was applied for 2 h, followed by three 5 min washes with TBST. Secondary antibody was put on for 30 min then washed three times for 5 min each with TBST. An Alexa conjugated secondary antibody (diluted 1:200 in serum) was put on the slide and incubated for 3 min. The slides were then washed three times for 5 min each and mounted using DAKO fluorescent mounting medium. The slides were then visualised either under fluorescent microscope.

RESULTS

Three cyclin kinase inhibitors p21, p27 and p53 were studied by immunocytochemistry (Fig. 1, 2, 3, 4).

The expression of cytoplasmic p21 increased ($p<0.05$) upon beta-1 integrin blocking at 120 h in culture. The expression of nuclear p21 increased ($p<0.05$) upon beta-1 integrin blocking at 72 and 120 h in culture. The expression of nuclear p21 increased ($p<0.05$) in culture from time 24 to 120 h upon beta-1 integrin blocking. A decrease ($p<0.05$) in the expression of cytoplasmic p27 was observed in cells from wt mice at 24 h in culture and in cells from p21 ko mice in all time points in culture, upon beta-1 integrin blocking. A significant increase ($p<0.05$) in the nuclear p27 expression was observed in cells from p21 ko mice at 24 h and in cells from wt mice at 72 h in culture upon beta-1 integrin blocking.

The expression of nuclear p53 increased significantly ($p<0.05$) in cells from wt and p21 ko mice upon beta-1 integrin blocking at 72 and 120 h in culture. Upon beta-1 blocking, there was an increase ($p<0.05$) in the expression of nuclear p53 from 24 to 120 h in culture in cells from both wt and p21 ko mice. No significant differences in the nuclear and cytoplasmic expression of p53 were noted in Clara cells from wt mice when compared to p21 ko mice. No significant changes in the expression of cytoplasmic p53 were observed upon beta-1 integrin blocking in cells from both wt and p21 ko mice.

DISCUSSION

Nuclear p21 and p53 expression increase upon beta-1 integrin blocking in clara cells culture: Upon beta-1 integrin blocking, an increase ($p<0.05$) in the nuclear p21 expression was observed in Clara cells. p21 expression was previously shown to be increased by the beta-4 cytoplasmic domain. Lower levels of $\alpha_6\beta_4$ surface expression were shown to induce p21 sufficient to induce partial G1 arrest and some apoptosis, while higher levels of $\alpha_6\beta_4$ expression resulted in more p21 expression and more widespread apoptotic death^[5]. Thus in this study, due to the fact that beta-1 integrin was blocked, there could have been a hypothetical increase in either the expression of $\alpha_6\beta_4$ or relatively more $\alpha_6\beta_4$ integrin adhered to an mice. The expression of cytoplasmic p21 increased ($p<0.05$) upon beta-1 integrin blocking at 120 h in culture [graph (A), control (not beta-1 integrin blocked) at 120 h (C), beta-1 blocked cells at 120 h (D)]. The expression of nuclear p21 increased ($p<0.05$) upon beta-1 integrin blocking at 72 and 120 h in culture (B) [graph (B), control (not beta-1 integrin blocked) at 120 h (C), beta-1 integrin blocked cells at 120 h (D)]. The expression of

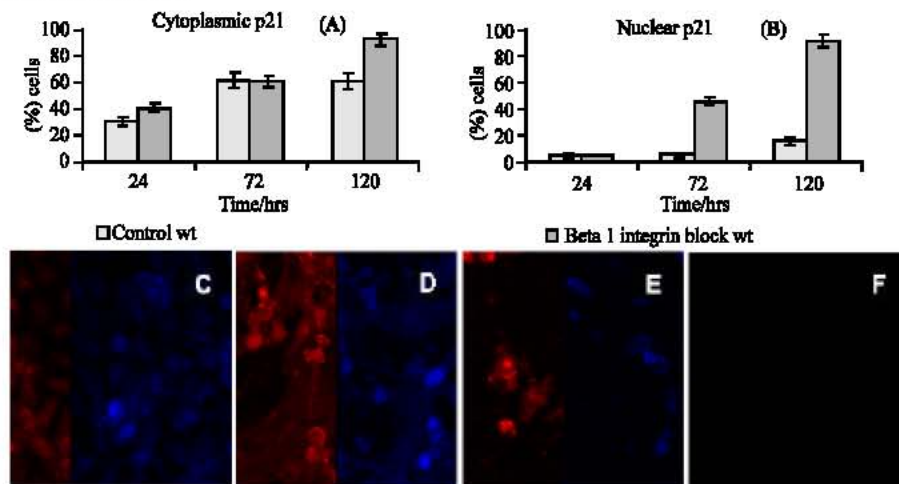


Fig. 1: Changes in the expression of p21 upon beta-1 integrin blocking in Clara cells from wt and p21 ko

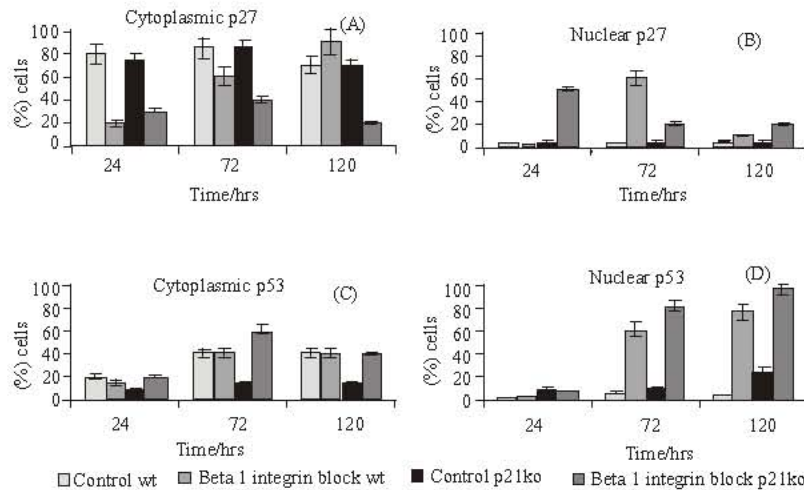


Fig. 2: Changes p27 and p53 expression upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice. Upon beta-1 integrin blocking, there was a decrease ($p < 0.05$) in the expression of cytoplasmic p27 was observed in cells from wt mice at 24 h in culture and in cells from p21 ko mice in all time point in culture (A). A significant increase ($p < 0.05$) in the nuclear p27 expression was observed in cells from p21 ko mice at 24 h and in cells from wt mice at 72 h in culture upon beta-1 blocking (B). No significant changes in the expression of cytoplasmic p53 were observed upon beta-1 blocking in cells from both wt and p21 ko mice (C). The expression of nuclear p53 increased significantly ($p < 0.05$) in cells from wt and p21 ko mice upon beta-1 integrin blocking at 72 and 120 h in culture. An increase ($p < 0.05$) in the expression of nuclear p53 was observed from 24 to 120 h in culture in cells from both wt and p21 ko mice (D)

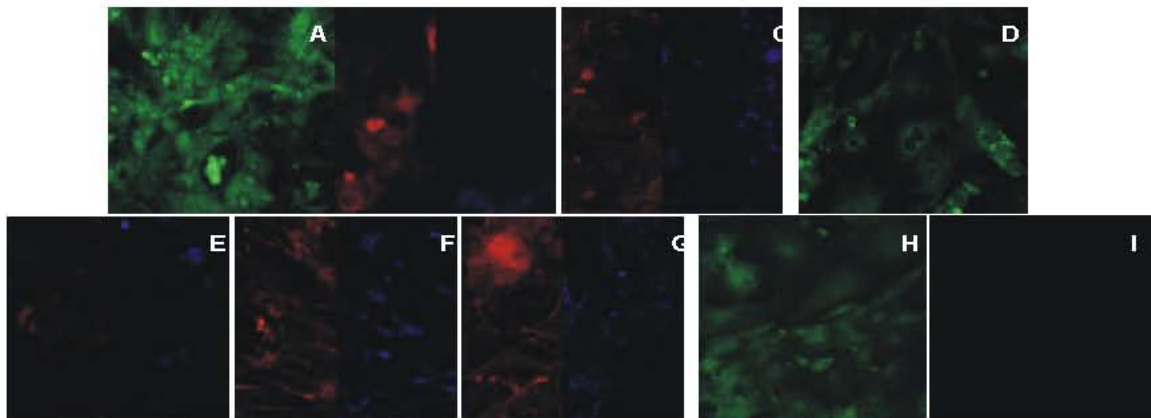


Fig. 3: Changes in p27 expression upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice. Upon beta-1 integrin blocking, there was a decrease ($p < 0.05$) in the expression of cytoplasmic p27 in cells from wt mice at 24 h in culture (A and B) and in cells from p21 ko mice at all time points in culture (cells from at 120 h in culture are represented in C and D). A significant increase ($p < 0.05$) in the nuclear p27 expression was observed in cells from p21 ko mice at 24 h (E and F) and in cells from wt mice at 72 h (G and H) in culture upon beta-1 blocking. [A, C, E and G are cells from control (not beta-1 blocked, while B, D, F and H are beta-1 blocked cells]. I represent the negative control for immunofluorescence whereby the primary antibody was omitted. Magnification $\times 200$

nuclear p21 increased ($p < 0.05$) in culture from time 24 to 120 h upon beta-1 integrin blocking [graphs (A and B), beta-1 blocked cells at 24 h (E), beta-1 blocked cells at 120 hr(D)]. F is a typical negative control for

immunofluorescence whereby the primary antibody was omitted. Magnification $\times 200$ appropriate receptor and thus results in an increase in p21. More work needs to be carried out, to find out the exact mechanism by which p21

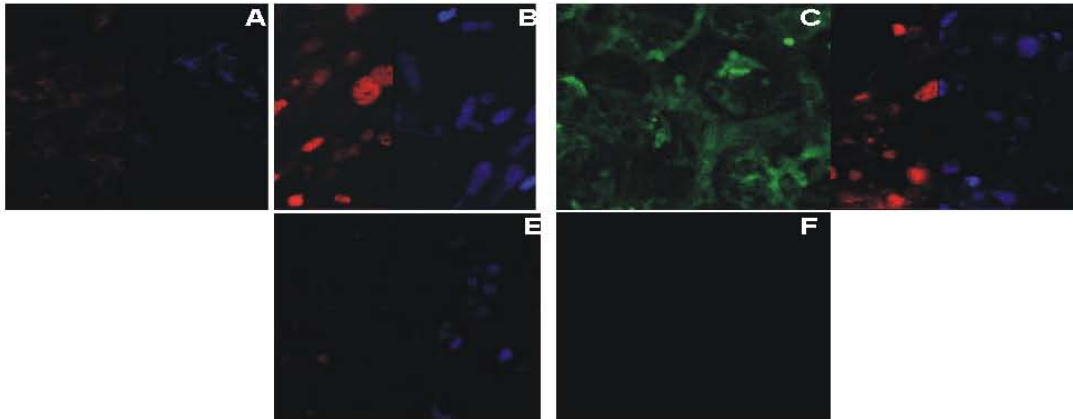


Fig. 4: Changes in p53 expression upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice. Upon beta-1 integrin blocking, the expression of nuclear p53 increased significantly ($p < 0.05$) in cells from wt mice (A and B) and in cells from p21 ko mice (C and D) at 120 h in culture. An increase ($p < 0.05$) in the expression of nuclear p53 was observed from 24 (E) to 120 (D) h in culture in cells from p21 ko mice. No significant changes in the expression of cytoplasmic p53 were observed upon beta-1 integrin blocking in cells from both wt and p21 ko mice (A - E). [A and C are cells from control (not beta-1 integrin blocked, while B, D and E are beta-1 blocked cells]. F is the negative control for immunofluorescence whereby the primary antibody was omitted. Magnification x 200

expression increases upon beta-1 integrin blocking. The literature about this topic is so far very limited. Upon beta-1 integrin blocking, the nuclear p53 expression increased ($p < 0.05$) in Clara cells from both wt and p21 ko mice. $\alpha_6\beta_4$ was shown to activate p53 Bachelder *et al.*^[8], whereas p53 itself was found to inhibit $\alpha_6\beta_4$ integrin survival signalling^[9]. The reason for the increase in the nuclear p53 could be the same as for the p21, but whether the increase of p21 is dependent or independent of p53 is not known.

In this study no significant differences in the nuclear and cytoplasmic expression of p53 were observed in Clara cells in the presence or absence of p21 upon beta-1 blocking. It seems that apart from beta-1 and beta-4 integrins, other integrins are involved, possibly beta-3, beta-6 or beta-7. Although beta-3 was found not to be present in lung epithelium cells^[15] but beta-3 expression was found to be present in the epithelial cells of fibrotic lungs^[19]. Beta-3 integrin was shown to be involved in the p53 and p21 expressions^[20,21]. α_v complexes either to β_3 , β_5 or β_8 were also found to be involved in the expression of p53 and p21^[22,23]. Thus, it can be hypothesised that the increase in p53 expression in the absence of p21 could be mediated via integrins, but further studies have to be carried out to determine which integrins are involved and to determine the exact mechanism.

P27 translocation to the nucleus occurs faster in the absence of p21 upon beta-1 integrin in clara cells: Upon

beta-1 integrin blocking, there was a decrease ($p < 0.05$) in the expression of cytoplasmic p27 at all time points in culture. An increase in nuclear p27 expression in cells from p21 ko mice at 24 h and in cells from wt mice at 72 h in culture, were observed upon beta-1 integrin blocking. Thus upon beta-1 integrin blocking, the translocation of p27 from the cytoplasm to the nucleus seems to be faster in the absence of p21. It has previously been hypothesised that p27 expression could be influenced via beta-1 integrin^[24-26], but the exact mechanism is still unclear and the literature so far is quite limited. Thus further studies need to be carried out to describe the relationship of p27 with integrins.

CONCLUSION

When cells are attached to $\alpha_6\beta_4$, p21 is not involved in the cytokeratins 8, 18 and 19 expressions of Clara cells, while when cell- $\alpha_6\beta_4$ interaction is disturbed the presence of p21 is important for the cytokeratins expression. Cell-beta-1 interactions could also be involved in the cytokeratins expressions. Therefore the expression levels of p21 could be a determinant factor in Clara cell differentiation. Cell-beta 1 interactions were described as a potential factor in cell proliferation. The lack of binding of beta-1 integrin to an appropriate receptor could lead to a decrease in cell proliferation. Beta-1 integrin disruption could also lead to an increase in PCNA expression but for an increase in nuclear PCNA p21 was shown to be an

essential factor. Upon blocking beta-1 integrin there is an increased nuclear expression of p21, p27 and p53 and an increase in apoptosis rate. Thus as originally hypothesised, integrins that bind to laminin are important for cell cycle progression through p21-dependent or -independent mechanisms.

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